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REPORT

RNA interference in human cells is restricted to the cytoplasm

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ABSTRACT

RNA interference (RNAi) is an evolutionarily conserved eukaryotic adaptive response that leads to the specific degradation of target mRNA species in response to cellular exposure to homologous double-stranded RNA molecules. Here, we have analyzed the subcellular location at which RNA degradation occurs in human cells exposed to double-stranded short interfering RNAs. To unequivocally determine whether a given mRNA is subject to degradation in the cytoplasm, the nucleus, or both, we have used the retroviral Rev/RRE system to control whether target mRNAs remain sequestered in the nucleus or are exported to the cytoplasm. In the absence of export, we found that the nuclear level of the RRE-containing target mRNA was not affected by activation of RNAi. In contrast, when nuclear export was induced by expression of Rev, cytoplasmic target mRNAs were effectively and specifically degraded by RNAi. Curiously, when the target mRNA molecule was undergoing active export from the nucleus, induction of RNAi also resulted in a reproducible approximately twofold drop in the level of target mRNA present in the nuclear RNA fraction. As this same mRNA was entirely resistant to RNAi when sequestered in the nucleus, this result suggests that RNAi is able to induce degradation of target mRNAs not only in the cytoplasm but also during the process of nuclear mRNA export. Truly nucleoplasmic mRNAs or pre-mRNAs are, in contrast, resistant to RNAi.

Keywords: RNA export; RNA interference; RNA stability; siRNAs

INTRODUCTION

Small interfering RNAs (siRNAs) are short double-stranded RNAs that can trigger the sequence-specific degradation of homologous mRNAs, a process termed RNA interference (RNAi; reviewed by Sharp, 2001). RNAi can be induced by direct transfection of siRNAs or by injection, transfection, or endogenous production of double-stranded RNAs that are then processed to siRNAs by the cell (Fire et al., 1998; Hamilton & Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001). RNAi has been observed in a wide range of species, including plants, nematodes, protozoa, insects, mammals, and at least some fungi (Fire et al., 1998; Ngô et al., 1998; Cogoni & Macino, 1999; Hamilton & Baulcombe, 1999; Caplen et al., 2000, 2001; Hammond et al., 2000; Elbashir et al., 2001). The conservation of RNAi through much of eukaryotic evolution clearly suggests that this adaptive response is of considerable importance. At present, RNAi is believed to primarily function as a cellular defense mechanism against viruses and transposable elements (Lindbo et al., 1993; Covey et al., 1997; Ketting et al., 1999; Tabara et al., 1999). However, processes related to RNAi also appear to be involved in the post-transcriptional regulation of genes important for metazoan development (reviewed by Ruvkun, 2001). The only current example of developmental regulation by small, noncoding RNAs involves the small temporal RNAs (stRNAs) let-7 and lin-4 first described in *Caenorhabditis elegans* (Lee et al., 1993; Reinhart et al., 2000). Of note, both siRNA and stRNA production requires the same cellular ribonuclease, a protein termed dicer (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Knight & Bass, 2001). Recently, a whole family of noncoding RNAs, structurally similar to *C. elegans* stRNAs, have been identified in cells from humans and other species (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001). Although the role of these so-called micro RNAs (miRNAs) re-

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mains to be determined, this discovery again suggests that RNAi and related phenomena are likely to be of general importance.

Although RNAi represents both an interesting phenomenon and a potentially invaluable experimental technique, many mechanistic aspects of RNAi remain poorly understood. Surprisingly, one question that has yet to be clearly resolved is the subcellular location at which RNA degradation occurs. Two lines of evidence suggest that RNAi is predominantly cytoplasmic. Thus, the enzymatic activity that mediates specific target mRNA degradation, an RNA-containing protein complex that has been termed the RNA-induced silencing complex (RISC), has been found to copurify with ribosomes (Hammond et al., 2000), thus suggesting that RISC might be localized to the cytoplasm. In addition, it has also been reported that dsRNAs targeted against intronic sequences are not effective inducers of RNAi (Fire et al., 1998; Ngô et al., 1998). On the other hand, it has also been shown that RNAi can significantly reduce the nuclear level of target mRNAs in *C. elegans* (Montgomery et al., 1998), a result that has been interpreted as indicating that RNAi can occur after pre-mRNA splicing but prior to nuclear mRNA export. Here, we demonstrate that mRNA molecules that are sequestered in the nucleus of human cells are not subject to RNAi.

RESULTS AND DISCUSSION

Cellular mRNAs can be divided, using biochemical techniques, into a cytoplasmic fraction and a nuclear fraction. However, the nuclear fraction consists not only of mRNAs located in the nucleoplasm per se, but also of mRNAs that are in the process of being exported (Hentze & Kulozik, 1999). Therefore, as these latter mRNAs are at least partly in contact with the cytoplasm, it is possible that a degradative process that is restricted to the cytoplasm would in fact result in a reduction in the operationally defined nuclear mRNA fraction. To avoid this potential artifact, we wished to examine the effect of RNAi on an mRNA that can either be sequestered in the nucleus or induced to enter the cytoplasm. One experimental system that permits the nuclear export of a specific mRNA to be regulated is the well-characterized Rev/RRE axis found in human immunodeficiency virus type 1 (HIV-1; reviewed by Cullen, 1998). In the absence of Rev, unspliced or incompletely spliced mRNAs encoded by HIV-1 are unable to leave the nucleus and accumulate there to readily detectable levels. However, expression of the viral Rev protein results in recruitment of Rev, together with the cellular nuclear export factor Crm1, to the *cis*-acting Rev response element (RRE) RNA target site, thus inducing the efficient nuclear export, and cytoplasmic translation, of these incompletely spliced viral mRNAs. Importantly, fully spliced HIV-1 mRNAs are, in contrast, exported from the nucleus even in the absence of Rev.

We have previously described the pgTat indicator construct (Malim et al., 1989), which contains the two coding exons of the HIV-1 Tat protein separated by an intron, derived from the HIV-1 *env* gene, that contains the RRE (Fig. 1A). In the absence of cotransfected Rev, this indicator plasmid expresses only a spliced cytoplasmic mRNA, encoding the full-length Tat protein. However, in the presence of Rev, the unspliced mRNA encoded by pgTat is able to exit the nucleus and is then translated to give a short form of Tat encoded solely by the first Tat coding exon (Fig. 1A).

The indicator construct utilized in this article, termed pgTat/dmNxt, was derived from pgTat by insertion of the 420-bp coding sequence of the *Drosophila nxt* gene (dmNxt; Wiegand et al., 2002) into the *env* derived intron (Fig. 1A). We have previously shown that transfection of an siRNA targeted to dmNxt results in the degradation of the endogenous dmNxt mRNA, and loss of dmNxt protein expression, in *Drosophila* S2 cells in culture (Wiegand et al., 2002). As shown in Figure 1B, this same siRNA also selectively blocked the expression of the short form of Tat, encoded by the Rev-dependent unspliced pgTat/dmNxt mRNA, when cotransfected into human 293T cells (compare lanes 4 and 5). Importantly, this siRNA did not affect Rev protein expression (Fig. 1B) and also did not block the expression of the unspliced mRNA encoded by the parental pgTat construct in the presence of Rev (data not shown). As predicted from earlier work (Fire et al., 1998; Ngô et al., 1998), this intron-targeted siRNA also did not significantly affect the expression of the full-length Tat protein encoded by the fully spliced pgTat/dmNxt mRNA (Fig. 1B).

We next quantified the effect of the dmNxt siRNA on nuclear and cytoplasmic expression of the spliced and unspliced mRNAs encoded by pgTat/dmNxt. As shown in Figure 2, and as expected, pgTat/dmNxt gave rise to readily detectable levels of both spliced and unspliced mRNA in the nuclei of transfected cells, but only the spliced mRNA was able to reach the cytoplasm effectively in the absence of Rev (lanes 2 and 3). In contrast, upon coexpression of Rev, both the unspliced and spliced mRNAs are efficiently exported (Fig. 2, lanes 6 and 7). Cotransfection of the dmNxt siRNA, in the absence of Rev, had no detectable effect on the nuclear level of spliced or unspliced pgTat/dmNxt RNA (Fig. 2, compare lanes 2 and 4) and, of course, did not reduce cytoplasmic spliced mRNA expression. However, when Rev was also expressed, cotransfection of the dmNxt siRNA resulted in the almost complete loss of cytoplasmic unspliced pgTat/dmNxt mRNA expression, although the spliced mRNA was not affected (Fig. 2, compare lanes 7 and 9). Remarkably, in the presence of Rev, this siRNA also resulted in a reproducible, approximately twofold drop in the nuclear level of the un-

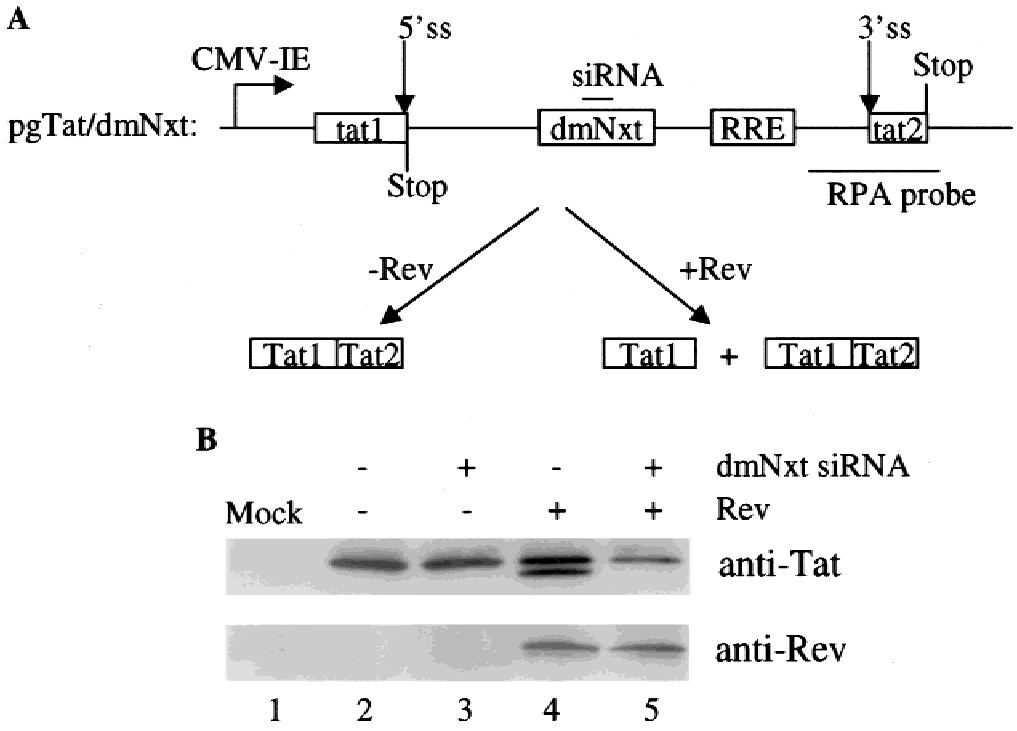

FIGURE 1. A: Schematic representation of the pgTat/dmNxt indicator construct. The relative positions of the two Tat coding exons, the splice sites (ss) and the Rev response element (RRE) are indicated. In the absence of Rev, only the spliced pgTat/dmNxt mRNA, encoding full-length Tat, is expressed. In the presence of Rev, the unspliced pgTat/dmNxt mRNA species is also exported from the nucleus, leading to expression of a short form of Tat. **B:** Western analysis of 293T cells transfected with pgTat/dmNxt in the presence or absence of pcRev and of the dmNxt-specific siRNA. The parental pBC12/CMV plasmid served as the negative control. The upper panel used a rabbit polyclonal anti-Tat antiserum and the lower panel, performed in parallel, used an anti-Rev antiserum.

spliced pgTat/dmNxt mRNA (Fig. 2, compare lanes 6 and 8).

The data presented in Figure 2 confirm earlier reports, derived from other species, demonstrating that RNAi results in the almost complete loss of the target mRNA in the cell cytoplasm and showing that dsRNAs targeted to introns do not affect the cytoplasmic expression of the cognate spliced mRNA (Fire et al., 1998; Ngô et al., 1998; Montgomery et al., 1998). However, Figure 2 also demonstrates an unexpected result, that is, when the unspliced pgTat/dmNxt mRNA was unable to exit the nucleus, it was not subject to any detectable degradation by RNAi. Conversely, when that same unspliced mRNA was being actively exported, due to coexpression of Rev, then ~60% of the operationally defined nuclear unspliced mRNA fraction became subject to degradation by RNAi.

As noted above, one possible explanation for this phenomenon is that the portion of the unspliced nuclear mRNA fraction that is subject to degradation is, in fact, in the process of being exported and is therefore not truly nuclear. This hypothesis is certainly consistent with the observation that all of the nuclear unspliced mRNA fraction is resistant to RNAi when it is sequestered in the nucleoplasm, away from the nuclear pores (Fig. 2). However, it is also possible to hypothesize that induction of RNAi, including nuclear RNAi, is primed by the presence of target mRNA molecules in the cytoplasm. Because the unspliced mRNA encoded by pgTat/dmNxt does not enter the cytoplasm when Rev is absent (Fig. 2), it could be argued that an effective RNAi response has not been induced in these cells.

To address this possibility, we constructed an expression plasmid encoding a fusion protein consisting of the dmNxt protein linked to the MS2 coat protein and to the transcription activation domain of VP16, which here serves as an epitope Tag. This fusion protein was expressed at readily detectable levels in transfected 293T cells, as determined by western blot analysis (Fig. 3A, lane 3). However, upon cotransfection of the dmNxt siRNA, MS2-VP16-dmNxt expression was essentially totally lost (Fig. 3A, lane 4). Therefore, an RNAi response specific for the dmNxt sequence has clearly been induced. However, as shown in Figure 3B, there is still no detectable effect of the dmNxt siRNA on the nuclear level of the unspliced mRNA encoded by the pgTat/dmNxt indicator plasmid in the absence of Rev (compare lanes 6 and 8).

Based on this result, we therefore conclude that RNAi in human cells can induce the degradation of mRNA molecules that are located in the cytoplasm. In contrast, mRNA molecules that are sequestered in the nu-

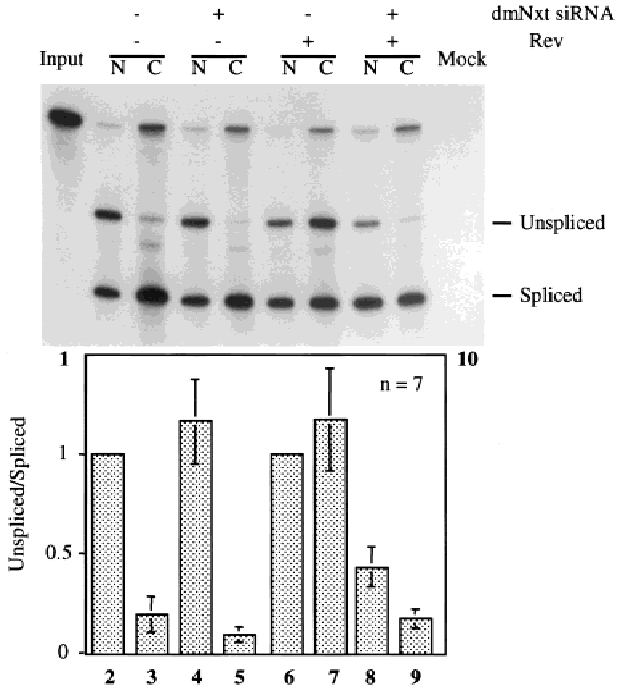

FIGURE 2. RNAse protection analysis of the level of spliced and unspliced pgTat/dmNxt mRNA present in the cytoplasm and nucleus of transfected 293T cells. Cells were transfected with pgTat/dmNxt in the presence or absence of pcRev, and in the presence of a dmNxt-specific or irrelevant siRNA, as indicated. Nuclear (N) and cytoplasmic (C) RNA fractions were isolated at 48 h after transfection and mRNA levels quantified using a previously described RPA probe (Kang & Cullen, 1999) that traverses the 3′ splice site of pgTat (Fig. 1A). The upper panel shows a representative RPA experiment and the aligned lower panel shows the average ratio of unspliced to spliced mRNA expression detected in each subcellular fraction, as quantified by PhosphorImager. An average of seven experiments with standard deviation is indicated. The ratio was arbitrarily set at 1.0 in lanes 2 and 6. The expression ratio in lanes 3 to 5 is given relative to lane 2, and the expression ratio in lanes 7 to 9 is given relative to lane 6.

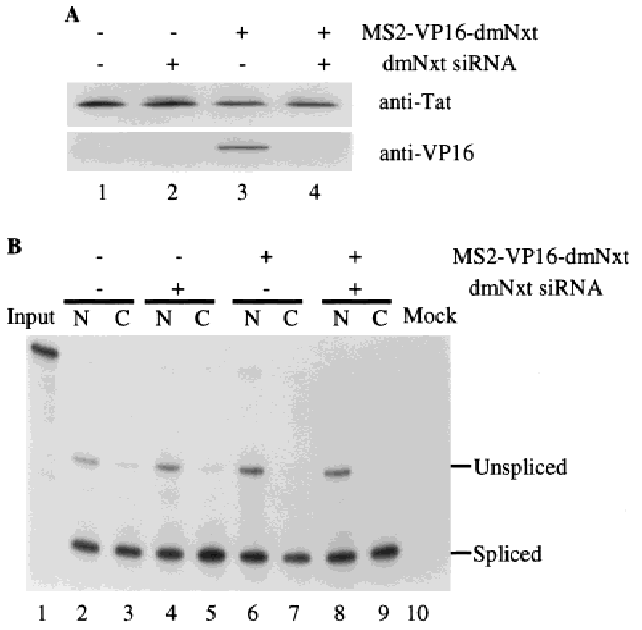

FIGURE 3. A: Western analysis of 293T cells transfected with pgTat/dmNxt in the presence and absence of pMS2-VP16-dmNxt and of the dmNxt-specific siRNA. Parallel immunoblots were performed using anti-Tat and anti-VP16 specific antisera. **B:** This RPA analysis was performed as described in Figure 2. See text for discussion.

cleoplasm are entirely resistant to RNAi. Thus, either the RNAi degradative machinery is absent from the nucleus, or these nuclear mRNA species are inaccessible for some other reason. These data are therefore consistent with earlier reports suggesting a cytoplasmic localization for RISC (Hammond et al., 2000) and demonstrating that nuclear pre-mRNAs are resistant to RNAi (Fire et al., 1998; Ngô et al., 1998). Data from studies in *C. elegans* documenting a partial decrease in the level of nuclear mRNAs during RNAi (Montgomery et al., 1998) could either be due to degradation of mRNA molecules caught during export, or might reflect a fundamental difference between humans and nematodes in the ability of RNAi to access the nucleus.

Although mRNA molecules sequestered within human nuclei were found to be entirely resistant to RNAi, ~60% of the biochemically defined nuclear fraction of this same mRNA became susceptible to RNAi when undergoing active nuclear export (Fig. 2). We hypothesize that these susceptible mRNA molecules are in the process of undergoing export, and therefore become accessible to the cytoplasmic RNAi degradative machinery, even though they remain part of the nuclear RNA fraction. However, we cannot formally exclude the possibility that a portion of truly cytoplasmic mRNAs localize to the perinuclear space and remain nucleus associated during the biochemical fractionation procedure. We note that the Tat protein does not contain a signal peptide and mRNAs encoding Tat should not, therefore, be selectively recruited to membrane-bound ribosomes.

It is interesting to compare the data reported in this article, analyzing the subcellular location of RNAi, with data from other groups examining the subcellular location of a genetically related process termed nonsense mediated decay (NMD; Domeier et al., 2000). NMD is a proofreading process that results in the specific destabilization of mRNA molecules that contain premature translation termination codons (reviewed by Maquat & Carmichael, 2001). Evidently, NMD requires the cell to first assess whether the translational open reading frame is intact, a process that presumably reflects mRNA translation by ribosomes. Indeed, NMD does not occur if translation is blocked. Surprisingly, however, NMD frequently results in a specific drop in not only the cytoplasmic level of the mRNA bearing the premature stop codon, but also in the nuclear level of that mRNA. Two hypotheses have been advanced to explain this observation (Hentze & Kulozik, 1999; Maquat & Carmichael, 2001). One proposal suggests that translation can, in

fact, occur in the nucleus, possibly in the form of a single "pioneer" round, and that this nuclear translation leads to nuclear NMD (Iborra et al., 2001; Ishigaki et al., 2001). Alternatively, it is known that mRNA molecules are exported from the nucleus cap first and that mRNA translation initiates prior to the complete transit of the mRNA through the nuclear pore (Daneholt, 1997). It has therefore been suggested that the drop in the nuclear mRNA fraction induced by NMD reflects degradation of mRNA molecules that are nucleus associated but accessible to cytoplasmic factors. Our observation that mRNA molecules that are sequestered in the nucleus are immune to RNAi, while identical mRNAs that are being actively exported from the nucleus are partially susceptible to RNAi (Fig. 2), suggests that cytoplasmic degradative pathways may indeed be able to target mRNA molecules that are in the process of leaving the nucleus, yet still form part of the nuclear mRNA fraction.

MATERIALS AND METHODS

Molecular clones

The parental expression plasmid pBC12/CMV and the derivatives pcRev and pgTat have been previously described (Malim et al., 1989). The pgTat/dmNxt plasmid was derived from pgTat by insertion of the *Drosophila nxt* coding sequence (residues 1 to 420, relative to the translation initiation codon) in place of a 580-bp *Bgl*II fragment excised from the pgTat intron. The *nxt* sequence was obtained by PCR amplification from a *Drosophila* embryonic cDNA library. The plasmid encoding the MS2-VP16-dmNxt fusion protein was prepared by insertion of the dmNxt coding sequence into a derivative of pBC12/CMV (Wiegand et al., 2002) encoding the MS2 coat protein RNA-binding domain fused to the VP16 transcription activation domain.

Western and RNAse protection assays

293T cells were maintained as previously described (Bogerd et al., 1995) and transfected using Lipofectamine 2000 (Invitrogen) in six-well plates. For the experiments shown in Figures 1B and 2, 0.1 μg of the pgTat/dmNxt indicator construct, 0.1 μg of pcRev, and $\sim$1 μg of siRNAs were used as indicated, whereas for the experiments in Figure 3, 0.2 μg of pMS2-VP16-dmNxt was also included. The dmNxt-specific siRNAs used in this report have been previously described (Wiegand et al., 2002) and are targeted to residues 184 to 204 in the dmNxt coding sequence. Importantly, this region of dmNxt is not conserved in either of the two human *nxt* genes.

Two days after transfection, the cells were harvested and a portion lysed for western blotting using polyclonal rabbit antisera directed against Tat or Rev (Malim et al., 1989), or using a monoclonal antibody specific for VP16 (Santa Cruz). The remaining cells were separated into cytoplasmic and nuclear fractions using an NP40 lysis procedure (Malim et al., 1989). Total RNA in each fraction was then isolated and analyzed by RNAse protection analysis (RPA) as previously described (Kang & Cullen, 1999).

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